Human Pathology (2018) 72, 107–116

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Nectin-4 promotes gastric cancer progression via the PI3K/AKT signaling pathway☆,☆☆

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Received 6 July 2017; revised 9 October 2017; accepted 18 October 2017

Keywords:
Nectin-4;
Gastric cancer;
Proliferation;
Migration;
PI3K/AKT pathway

Summary Nectin-4, a member of the Nectin family that includes 4 Ca^{+}-independent immunoglobulin-like cell adhesion molecules, plays a carcinogenic role in multiple cancers. However, Nectin-4 expression and its biological role in gastric cancer (GC) remain largely unknown. In this study, quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemistry were used to evaluate the expression patterns of Nectin-4 in GC specimens and cell lines. We observed that high expression of Nectin-4 in GC patients was associated with TNM stage and lymph node metastasis status, and poor prognosis. In addition, cell proliferation and cell migration assays in vitro and tumorigenicity in vivo were performed to observe the effects of up-regulation and down-regulation of Nectin-4 expression on GC cell phenotypes. In further studies, the PI3K/AKT signaling pathway was revealed to be involved in Nectin-4–mediated GC progression. These results demonstrated that Nectin-4 had a promoter effect on human GC cell growth and motility, indicating that Nectin-4 may serve as an effective therapeutic target in GC.

1. Introduction

Gastric cancer (GC), one of the most fatal malignancies around the world, is considered the second leading cause of cancer-related death in China [1]. The recommended treatments for GC are surgical operation, adjuvant therapy chemotherapy, radiotherapy, and molecular targeted therapy [2]. Although numerous advances have been made in diagnosis and therapeutic measures, the overall 5-year survival rate of patients with advanced GC remains at 25% [3]. Therefore, identification of new molecular biomarkers and cancer therapeutic targets can improve the prognosis in GC patients.

The Nectin family comprises Ca^{2+}-independent immunoglobulin-like cell adhesion molecules and includes 4 members (Nectin-1, Nectin-2, Nectin-3, and Nectin-4), which are involved in several functional processes, such as cell adhesion, movement, proliferation, differentiation, and polarization [4]. Nectin-1, Nectin-2, and Nectin-3 are broadly expressed in normal adult tissues, whereas Nectin-4 is expressed uniquely in the embryo and placenta [5]. A growing number of studies indicate that Nectin-4 may be regarded as a potential target for cancer therapy in many human cancers [6-8]; however, the expression of Nectin-4, as well as its prognostic

☆ Competing interests: The authors declare no conflict of interest.
☆☆ Funding/Support: These studies were supported by the National Natural Science Foundations for Young Scientists of China (Grant No. 81502053).
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https://doi.org/10.1016/j.humpath.2017.10.034
0046-8177/

significance and molecular mechanisms in GC, has not been clearly illuminated.

Rac1, a member of the Rho family GTPases, which play a vital role in various types of cellular processes, is closely correlated with the occurrence and development of many cancers [9,10]. Rac1 GTPase switches responsiveness to a GDP-bound form (inactive) to a GTP-bound form (active) and interacts with its effectors to participate in downstream cellular functions [11]. Once activated, Rac1 promotes the extension of several protrusions, especially lamellipodia, acting as a considerable driving force to cell motility [12,13].

Recent studies have demonstrated that the PI3K/AKT pathway is the major signaling pathway regulating Rac1 activity in many cancers [14,15]. Whether or not this pathway plays a role in Nectin-4/Rac1–mediated cell progression in GC remains to be determined. In the current work, we aim to validate the hypothesis that Nectin-4 promotes GC progression via the PI3K/AKT pathway.

In this study, we analyzed Nectin-4 expression in GC by quantitative real-time polymerase chain reaction (qRT-PCR), Western blotting, and immunohistochemistry (IHC), and investigated its relationship to clinical parameters and its prognostic significance. We further analyzed whether different expressions of Nectin-4 promote GC cell proliferation and migration, and in particular, the signaling pathways involved in the oncogenic function of Nectin-4–mediated GC cell progression. Our findings represent new understanding of the molecular mechanisms and therapeutic treatments for GC.

2. Materials and methods

2.1. Compliance with ethical standards

The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University. All procedures performed in this study were in accordance with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients included in the study.

2.2. Tissue specimens and cell lines

The paired GC tissues and adjacent nontumor tissues were obtained from 64 GC patients who underwent radical surgical resection at the Department of General Surgery, Affiliated Hospital of Nantong University. All patients were reevaluated pathologically for grade and histologic type according to the criteria of the American Joint Committee on Cancer by 2 independent pathologists. Tissue specimens were flash frozen immediately after surgery and stored in liquid nitrogen until protein and RNA extraction.

The human GC cell lines (SGC7901, AGS, MKN45, BGC823, MKN28) and normal human gastric epithelial cells (GES-1) used in this study were purchased from American Type Culture Collection (Manassas, VA) and were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, streptomycin (50 μg/mL), and penicillin (50 U/mL), and propagated in a 5% CO_2, 37°C humidified incubator.

2.3. Quantitative real-time PCR

Total RNA from frozen tissues and cell lines was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA), and then was reverse transcribed into cDNA using Primescript RT Reagent (Takara, Tokyo, Japan). qRT-PCR assays were carried out to detect messenger RNA (mRNA) expression levels using SYBR Premix Ex Taq (Takara) according to the manufacturer's protocol. Nectin-4 primers used in qRT-PCR were as follows: forward 5′-CAAAATCTGTGGCACATTGG-3′and reverse 5′-GCTGACATGGCAGACGTAGA-3′. β-Actin mRNA levels were used to normalize Nectin-4 target gene expression using the following primers: forward 5′-AGA GCCTCGCCTTTGCCGATCC-3′ and reverse 5′-CTGGG CCTCGTCGCCCACATA-3′. All procedures were performed in triplicate.

2.4. IHC staining

IHC analysis was conducted as previously described [16]. The immunoreactive score (IRS), which assessed the percentage of positive cells and the staining intensity, was used to analyze the IHC staining. Staining intensity was classified as (i) no staining (0), weakly positive (1), moderately positive (2), and strongly positive (3). The portion of Nectin-4–stained cells (ii) was scored as 0 (negative), 1 (1%-10%), 2 (11%-50%), and 3 (>50%). Both of them were multiplied as (i) × (ii), and the optimal cutoff value for IRS was determined as 3. Hence, the values of the samples with IRS ≥ 3 were categorized as high expression of Nectin-4, and the values less than 3 were regarded as low Nectin-4 expression.

2.5. Construction of recombinant plasmids

The full-length open reading frame of Nectin-4 was amplified from the human cDNA library (GenBank: NM_030916.2) using PrimeStar PCR and constructed into the expression vector pcDNA3.1B to generate pcDNA3.1B–Nectin-4. The sequence of the forward primer was 5′-EcoRI-AGAGAATTC ATGCCCCTGTCCCTGGGAGCC-3′, and the sequence of the reverse primer was 5′-BamHI-AGAGGATCCTCAGAC CAGGTGTCCCCGCCC-3′. The construct was verified by sequencing.

2.6. Short hairpin RNA preparation

Small interference (si) RNAs against Nectin-4 were designed according to Invitrogen and chemically synthesized by Shanghai